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14. ABSTRACT <b>Background:</b> Schwannomatosis is a recently recognized third major type of neurofibromatosis. Our preliminary studies of the <i>NF2</i> gene in tumors from schwannomatosis patients reveal a pattern of tumor suppressor gene inactivation not previously reported in any other human disease. <b>Objective/Hypothesis:</b> The objective of this project is to clone the locus responsible for familial schwannomatosis. We are exploring two competing hypotheses which address both the non random distribution of LOH observed in schwannomatosis tumors and the high rate of somatic <i>NF2</i> mutation seen along the <i>cis</i> allele. <b>Specific Aims:</b> 1. To identify and clinically characterize schwannomatosis patients, and maintain a resource of tumor and blood specimens. 2. To further refine the candidate region on chromosome 22 using linkage and loss of heterozygosity analyses. 3. To determine the molecular mechanism of tumor formation in these patients using complementary molecular and cytogenetic approaches. <b>Study Design:</b> Schwannomatosis patients and affected relatives will be identified.					
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## Introduction

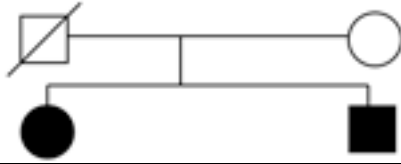
Neurofibromatosis (NF) encompasses a diverse group of genetic conditions whose common element is tumors of the nerve sheath. Schwannomatosis is a recently recognized third major type of NF, which results in multiple schwannomas without vestibular tumors diagnostic of NF2. Recent epidemiological studies have shown that schwannomatosis is as common as NF2. Our preliminary studies of the *NF2* gene in tumors from schwannomatosis patients revealed a pattern of tumor suppressor gene inactivation not previously been reported in any other human disease. The objective of this project is to clone the locus responsible for familial schwannomatosis, *sch*. We are exploring two competing hypotheses which address both the non random distribution of LOH observed in schwannomatosis tumors and the high rate of somatic *NF2* mutation seen along the *cis* allele. The first hypothesis is that *sch* is a second tumor suppressor gene which lies near to *NF2* on chromosome 22. In this model, schwannoma formation is dependent on four “hits” (two in the *sch* tumor suppressor, and two in the linked *NF2* tumor suppressor). FISH results have suggested a second hypothesis in which a structural element facilitates loss of the *trans* chromosome by increasing the rate of mitotic recombination. This is an especially attractive hypothesis since rates of mitotic recombination are both highly variable and genetically determined in humans.

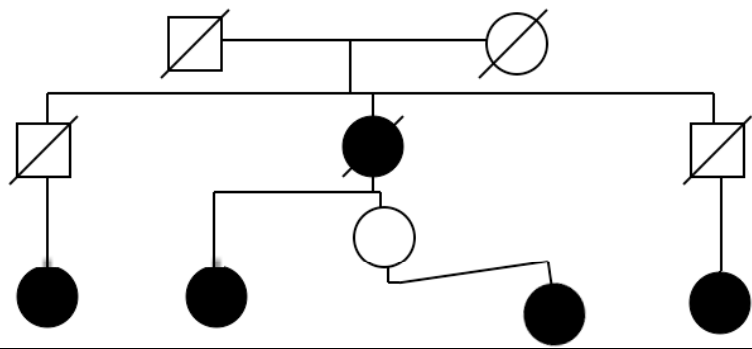
## Body

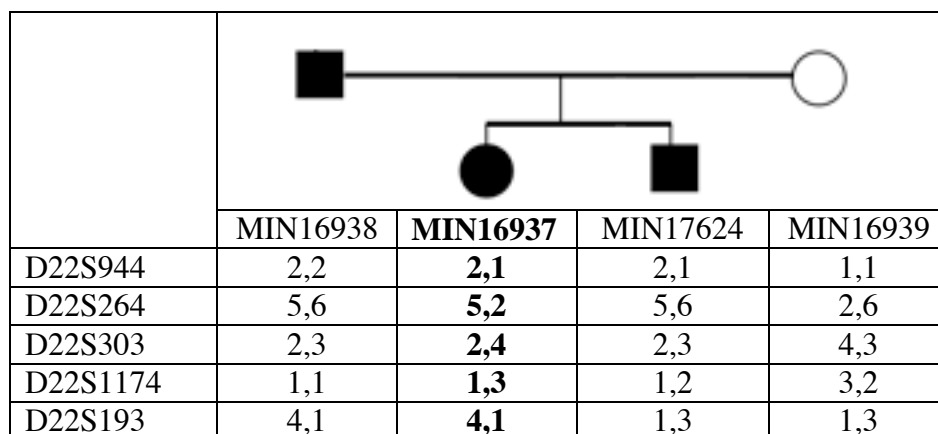
This section is organized around the approved statement of work.

*Task 1. To develop a resource of study subjects and related biological materials (months 1 through 30):*

Over the past year we have developed an unprecedented resource of human hybrid cell lines to enable the sequencing effort described in Task 3.e. below. The clear advantage of using hybrid material which contains only a single and mutation bearing chromosome is that deletions and re arrangements which do not involve duplication will be directly detected in sequencing if they are relatively small and will be detected by lack of PCR product if they are large. Similarly, careful placement of overlapping primers will detect insertions of material not otherwise in the candidate region directly if they are small and indirectly through PCR failure if they are large. We have developed hybrids from non founders only to exclude the possibility of mosaicism. There remains the possibility that sequential duplication which preserves a copy of all normal material will be missed by this method, however unless the duplication has independently arisen multiple times and is identical in unrelated non founders it is highly unlikely that its organization will preclude detection in multiple cell lines. The material used in developing these lines is shown in figure 1.

				
	<b>GUS27918</b>		<b>GUS19612</b>	
	Blood	Tumor (B1401)	Blood	Tumor (DAV153)
D22S427	3,2	3, 0	3,2	2,0
D22S264	6,3	6,0	6,3	6,0
D22S1174	3,1	3,0	3,1	3,0
D22S193	2,3	2,0	2,3	2,0
D22S929	3,1	3,0	3,1	3,0
<i>NF2</i> mutation	---	exon 10 FS	---	exon 1 FS

							
	GUS 29211	GUS 22693	GUS 29190	GUS 29189	<b>GUS28235</b>		
					Blood	Tumor (B1537)	Tumor (B1539)
D22S427	2,3	2,1	2,1	2,3	2,3		2,0
D22S264	1,2	1,3	1,3	1,3	1,5	1,0	
D22S1148	3,3	3,3	3,3	3,1	3,3		
D22S1167	4,2	4,4	4,4	4,2	4,2	4,0	
D22S193	2,2	2,5	1,5	1,4	2,2		
D22S430	4,2	2,1	3,1	3,3	4,2	4,0	4,0
<i>NF2</i> mutation						exon 11, FS	exon 6, NS



**Figure 1. Material used to derive somatic cell lines.** Three pedigrees from family 1 (top), 10 (middle) and a segment of PA-3 (bottom) are shown above a selected group of anonymous microsatellite polymorphisms which are positioned around the candidate region. Haplotypes at each marker are listed for affected and obligate carriers within each pedigree. Retained alleles in tumors with loss of heterozygosity are shown next to the paired blood specimen. The first line is derived from subject GUS19612 who shares both an affected and unaffected chromosome 22 with his affected sister from the proximal border of the candidate region at D22S264 through the *NF2* region at D22S193 and D22S929. Both individuals are polymorphic at all of these markers and both phase through the region by tumor tissue with loss of heterozygosity. The second line is derived from subject GUS28235 who shares the region from D22S427 through D221167, but not distally in the *NF2* region itself; her affected chromosome is also defined by LOH in two tumor specimens. In family PA-3, MIN16937 shares a chromosome with her affected brother and father; she carries the opposite unaffected maternal chromosome from her brother. FS = frameshifting mutation. NS = nonsense mutation. Absolute genomic position and primer placement for these markers available through the resources of the NCBI: <http://www.ncbi.nlm.nih.gov/>

To date we have developed and validated human hamster hybrid lines from family 1 and PA-3 using a non selection somatic cell fusion of EBV transformed peripheral lymphocytes to CHTG 49. Phasing with family and tumor material has allowed us to select two clones containing only an affected chromosome 22 on hamster background (family 1, clone 37; family PA-3 clone 4) and as control material two clones containing only an unaffected chromosome 22 (family 1 clone 21 and family PA-3 clone 9). Initial sequencing results from task 3e have confirmed these phasings. In conjunction with Dr. Robert Jenkins of the recently available Conversion Technology Laboratory at the Mayo Clinic, a complementary second set of hybrid lines from family 11 and PA-3 has been developed. These lines were fused to a mouse background, and individual colonies picked from the Petri dishes into a 96-well microtiter plate. After screening by high throughput genotyping, those clones containing a single chromosome 22 were selected for expansion. We are currently confirming the initial genotyping on four putative affected chromosome bearing lines (two from family PA-3 and two from family 11) and five putative unaffected chromosome bearing lines as controls (two from family 11 and three from family PA-3).

In addition the following progress has been made on the other subgoals of this task:

*b. Obtain specimens from confirmed probands and affected relatives (months 6 through 30).*

A total of 10 blood specimens were collected from newly identified familial clusterings, newly identified sporadic cases and expansion of previously identified familial clusterings. 15 flash frozen tumor specimens from clinically indicated surgery were obtained.

*c. Perform NF2 mutational analysis on selected tumor specimens from confirmed probands (months 12 through 30).*

We have now completed *NF2* mutational analysis on 52 tumors from familial schwannomatosis patients. These tumors derive from 29 study subjects in 15 unrelated families and include two meningiomas (one found incidentally at autopsy) and one mesothelioma (also derived from an autopsy case). The spectrum of mutations in these tumors is quite striking. Overall, mutations were detected in 34 of 49 schwannomas (69%), a detection rate somewhat lower than seen in our studies of sporadic schwannomas (86%, Jacoby et al., 1996). Of the 32 mutations easily classified as to effect, 15 involved frameshifting deletions/insertions (47%), 11 nonsense changes (34%, including a six basepair deletion resulting in formation of a nonsense codon at the junction site) and 6 splice site changes (19%). Compared to literature reports of mutation in sporadic schwannomas (227 total mutations, summarized with references in Ahronowitz et al., in press) this reveals a significant shift towards nonsense mutation (from 19% in sporadic tumors) and away from frameshift mutations (from 62% in sporadic tumors) with a lack of non truncating mutations that may be due to small sample size (only 4% in sporadic tumors). Furthermore, although nearly three quarters of nonsense mutations in sporadic tumors can be attributed to C to T transversion at CpG dinucleotides, only 3 of 11 (27%) of schwannomatosis tumors were so mediated. This larger dataset continues to support the unusual and unexplained observation of a paucity of mutation in the alpha helix domain of the protein as noted in last year's progress report.

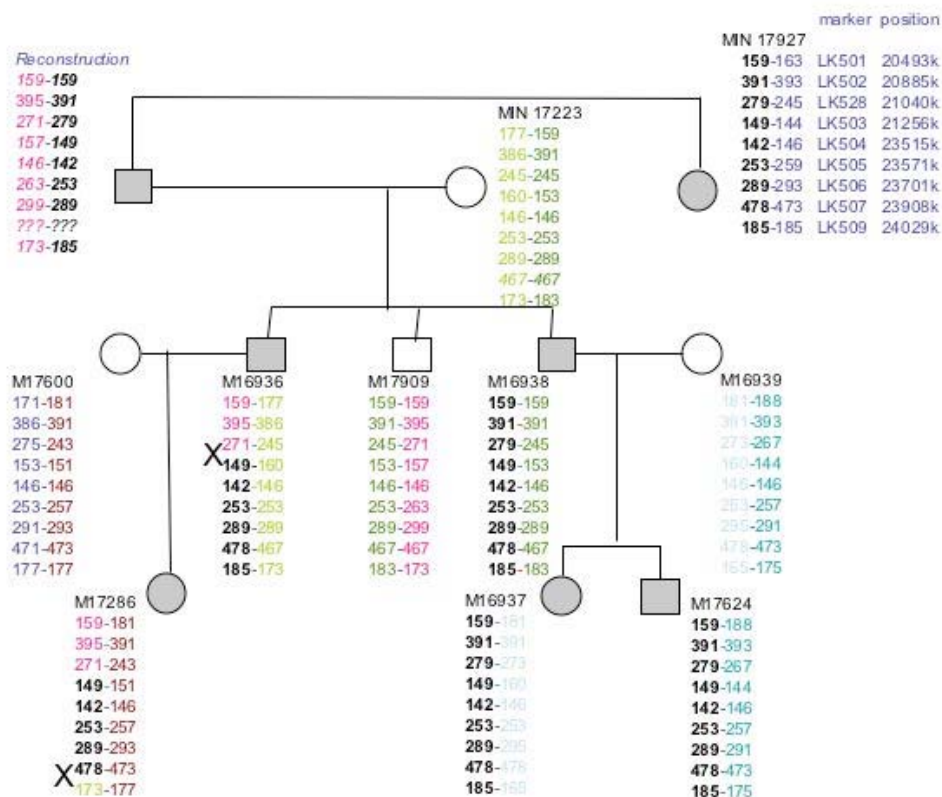
*Task 2. To refine the candidate region using LOH and linkage analysis (months 12 through 36):*

*a. Perform LOH analysis on tumor specimens from confirmed probands, including 10 paraffin blocked specimens and 6 frozen tumors per 12 month time period (months 12 through 36)*

LOH analysis at microsatellite markers has been completed on the same set of 52 familial schwannomatosis tumors described above. LOH of *NF2* region markers was seen in 39 of 52 tumors (75%), a significantly higher percentage than our similar studies of sporadic schwannomas (50%, Jacoby et al., 1996). Somewhat inexplicably, although LOH is clearly observed at microsatellite markers in a majority of tumors, we have observed that often direct sequencing of mutations does not appear to show LOH (further explored in section 3d). Although we had previously hoped that LOH patterns might refine a candidate region of interest, we have found that family studies are a much more powerful method for doing so.

*b. Perform linkage analysis on newly identified families, including 2 to 3 affected kindreds in each 12 month time period (months 12 through 36). and c. Interim analysis and publication refining the candidate region at months 24 to 27.*

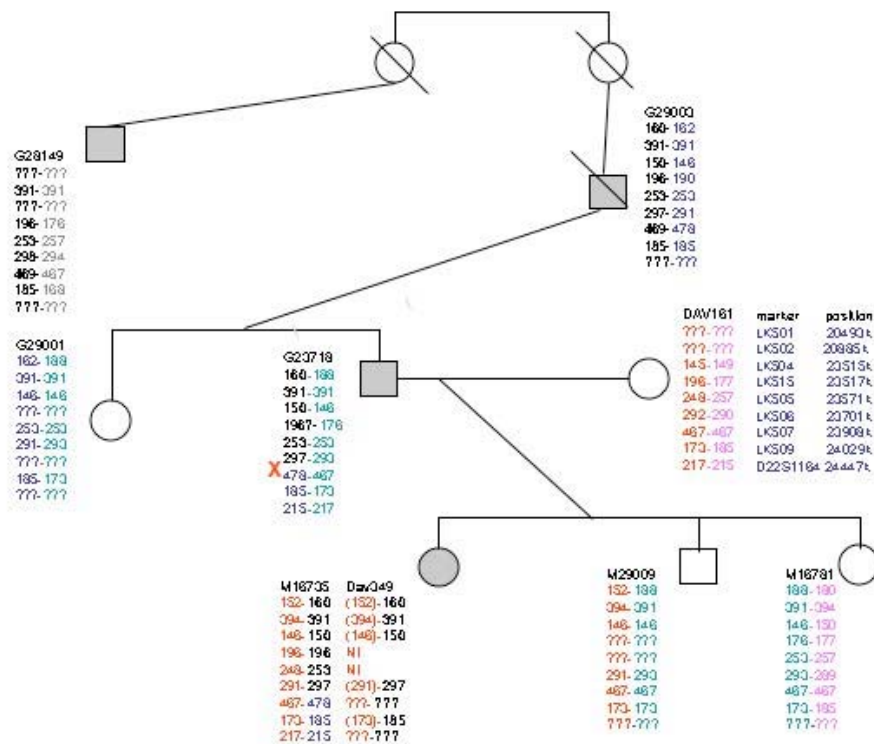
Over the past year, we have continued to focus on refining our best evidence candidate region. The centromeric border is now defined by an indirectly observed cross in family PA-3 between our newly generated marker LK528 (at 21,040k) and LK503 (at 21,256, figure 2).



**Figure 2. Centromeric border of the candidate region.** Absolute allele size in a portion of family PA-3 is shown. **Black bold** signifies the affected allele with M17624 defined as the proband. Alleles in italics are inferred. Positions of these newly developed markers on the long arm of chromosome 22 in build 35.1 is given at the top right of the figure.

Although a telomeric cross is also seen in family PA3 between markers LK507 and LK509 (figure 2), the telomeric border is now defined by a directly observed cross in family 9. This occurs between marker LK506 (at 23,701k) and LK507 (at 23,908k, figure 3). To further confirm the candidate region and to exclude the possibility of linkage heterogeneity, all seven multigenerational/informative families have been haplotyped at the markers D22S303 (chromosome position 21,593k) and AB05 (22,363k) and shown to share an allele between all affected family members.





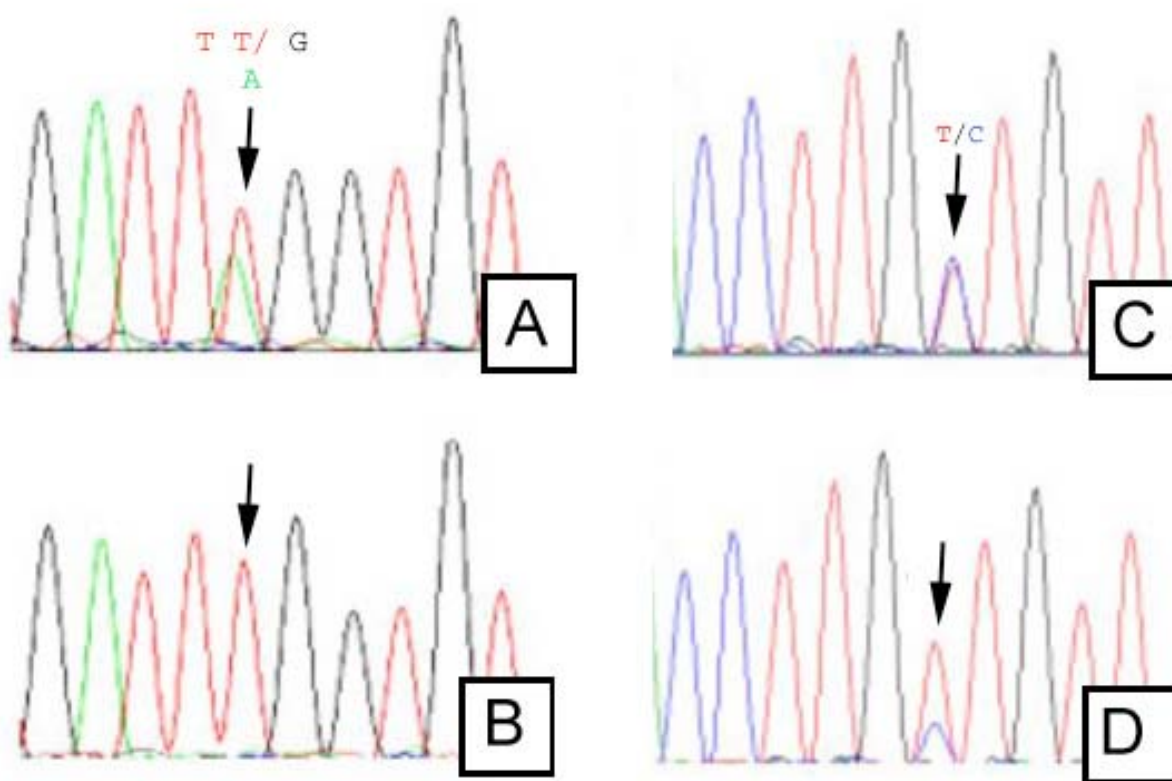
**Figure 3. Telomeric border of the candidate region.** Absolute allele size in a portion of family 9 is shown. **Black bold** signifies the affected allele with G29003 defined as the proband. Alleles in parenthesis in tumor sample DAV349 are lost compared to the paired blood sample M16735. Positions of these newly developed markers on the long arm of chromosome 22 in build 35.1 are given in the middle right of the figure. Note that LK501/LK502 and S1164 are widely flanking the central core of the clustered markers.

*Task 3. To determine the molecular mechanism leading to schwannomatosis (months 1 through 48):*

*d Application of FISH probes to schwannomatosis related tumors to determine mechanism of LOH (months 30 through 45).*

Our previous application of FISH probes to schwannomatosis related tumors has suggested that isodisomy is the major mechanism of LOH and the work of others has suggested that this may be more widely applicable to non vestibular tumors in general. Over the past year we have begun to extend these observations using the recently published HapMap database (<http://www.hapmap.org/>, International HapMap Consortium, 2005) to develop resources with in the *NF2* locus that could be directly compared to the acquired mutations in these tumors. Specifically, we sought to determine the reason for the apparent contradiction between sequencing results outlined in section 1.c. above and LOH results using microsatellite markers outlined in section 2.a. above (figure 4A). We indeed confirmed that although the mutation itself

frequently does not show the expected loss of the wild type allele, embedded SNPs frequently do (figure 4B, and table 1). To explain these results, we are currently exploring two competing hypothesis, namely that somatic mutation in the *NF2* gene in schwannomatosis tumors occurs before LOH implying that haplo insufficiency is sufficient for tumor formation in the face of bi allelic change at *sch* or that schwannomatosis tumors (like those of NF1 patients) consist of a genetically heterogeneous population of cells.



**Figure 4. Loss of heterozygosity patterns in familial schwannomatosis tumors.** A. Tumor DAV382 derived from a non founding member of family V carries a nonsense T to A change (arrow) in codon 49 of exon 2 causing a change from TTG (leu) to TAG (stop). The wildtype T allele is slightly larger then the mutant A allele, suggesting that there is slight contamination of normal tissue within a tumor that has not lost heterozygosity. Note that as described in section 2c, this nonsense mutation is somewhat atypical in that it does not occur at a CpG island. B. As expected, there is no change in the paired blood sample to this tumor which shows only wildtype sequence (arrow). The peak of the T allele is higher then that shown in part A, suggesting one copy of the T allele in the tumor and two copies in the blood as would be expected. C. SNP2250752, embedded in the *NF2* gene, is present in the blood sample as the recorded T/C polymorphism (arrow). The allele peak is exactly equal as expected. D. SNP2250752 in the tumor sample shows clear loss of the C allele (arrow) with a small amount of residual peak of the T. This is consistent with loss of heterozygosity of the T allele with a small amount of normal tissue contamination and seems to conflict with the results of panel A.

Family	sample ID	WT <i>NF2</i> to mutation in tumor (percent is estimate only)	estimated amount of reduction of one SNP allele compared to other in tumor*	notes
11	247	equal	>50%	
NH	371	less (33%)	33%	<b>EXPECTED</b>
E	378	more (75%)	25%	
P	377	more (80%)	close to 100%	
V	380	more (75%)	75%	
V	382	more (10%)	80%	
V	342	more (20%)	85%	
V	367	less (25%)	75%	<b>EXPECTED</b>
<b>expected if LOH event</b>		less (100% = no normal tissue contamination)	100% = no normal tissue contamination	
<b>expected if no LOH event</b>		equal	equal	

**Table 1. Summary of LOH patterns in familial schwannomatosis tumors.** Eight tumors known to have single basepair changes for which frozen tumor was available were analyzed at one or more known SNPs. In six of eight cases the pattern seen at the SNP was similar to that seen in microsatellite markers and seemingly contradicted the pattern seen at the mutation itself. \*all SNPs were close to 50:50 in blood (figure 4c). SNP alleles in red were higher than in blood sample suggesting isodisomy event (figure 4D). The wildtype *NF2* allele was never higher in tumor than in blood.

*e. Screening of candidate regions and/or loci based on results of task 2 (months 30 through 45).* Because of our previous negative results regarding expressed elements in this region and the similar negative results from Dr. Dumanski's laboratory reported last year (Buckley et al., 2005), we have begun a genomic sequencing approach to the current candidate region which stretches from the centromeric marker LK528 (at 21,040k) to the telomeric marker LK507 (at 23,908k), approximately 3 Mb. All primary sequencing is being carried out in the somatic cell hybrids described in task 1, consisting of the affected/transmitted chromosome 22 from a non founding affected individual on a hamster or mouse background. We plan to seed the region at 10 roughly equally placed start points and perform unidirectional PCR based sequencing of approximately 400 basepairs per reaction overlapping with adjacent amplicons by 50 basepairs. Alterations from NCBI deposited sequencing in the current build will be detected using an automated package of variant identification software such as the Sequencher suite of programs ([www.Sequencher.com](http://www.Sequencher.com)). Ambiguous sequencing will be repeated from the same or opposite direction, or in selected cases, from the rodent background. Alterations from the NCBI consensus sequence and known human polymorphisms will immediately be confirmed in affected family member(s) and subsequently the region will be examined in a panel of 17 affected unrelated non founders. As finalization nears, sequence is maintained on a secure website (<http://schwannomatosisproject.mgh.harvard.edu>). Over the past year, we have begun work on 2 of 10 seeding locations and in one of these two identified a three previously un

recognized low copy repeats (LCR) which span the 3' end of the candidate region. A translocation between two of these repeats has occurred on the affected allele of family 1, however it is also seen in 4% of unaffected alleles tested and therefore we have excluded it as causative. Family 1 also carries a total of 8 alterations from wild type sequence in one 200 basepair region of the second LCR. These changes are not seen in other affected families, nor in a panel of 50 unaffected unrelated unaffected individuals. We are currently working to understand potential effects of these changes in a non coding region on adjacent transcripts.

## Key Research Accomplishments

- Development and validation of human hybrid cell lines containing affected chromosomes from non founding schwannomatosis families.
- Completion of molecular analysis of the *NF2* region in a large cohort of familial schwannomatosis tumors with documentation of significant molecular differences from sporadic tumors.
- Refinement of the schwannomatosis candidate region to the region from 21,040k to 23,908k.

## Reportable Outcomes

### *Presentations:*

Invited presentations by the PI concerning this research were made at the 11<sup>th</sup> European Neurofibromatosis meeting (Gotenborg, 7/05), Neurology Grand Rounds (Mass General Hospital, 9/05), the Neurofibromatosis Society of Ontario (Toronto, 10/05), the Children's Tumor Foundation (Banbury, 11/05), Neurosurgical Grand Rounds (Mass General Hospital, 1/06), Brown Neurology/Child Neurology Grand Rounds (Providence, 3/06) and the Molecular Neurogenetics Unit (Mass General Hospital, 4/06).

An invited presentation by Dr. Lan Kluwe concerning this research was made to the Children's Tumor Foundation (6/06).

### *Funding applied for:*

The PI supported the submission of a proposal on "Molecular analysis of clinical subsets of schwannomas" by Dr. Anat Stemmer-Rachamimov to the NIH in February, 2006. Although this proposal was not funded, the PI is currently working with Dr. Stemmer on a revision.

The PI supported the submission of a reply to the NIH initiative for large scale genomic sequencing (NOT-HG-05-006) in November, 2005 by Dr. Lan Kluwe. The response to this request is pending.

### *Research opportunities:*

The PI sponsored Dr. Lan Kluwe as a visiting scientist in the Department of Neurology at Mass General Hospital, October, 2005 to present.

The PI sponsored Dr. Marcelo Paula Coutinho as a visiting post doctoral fellow from the University of Teresopolis, Rio de Janeiro Brazil; "Mutational analysis of *CABIN1* in a cohort of schwannomatosis nonfounders," September, 2005 to December, 2005.

## Conclusions

Schwannomatosis is a third major form of NF, which recent epidemiological studies have shown is as common as NF2. However, clinical recognition and molecular characterization have lagged far behind other forms of NF. The clarification of molecular alterations in schwannomatosis will likely have broad implications for other tumor suppressor gene syndromes. We have identified significant resources over the past year to aid in the search for the fundamental alteration in schwannomatosis.

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